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ACETYLCHOLINE RECEPTOR BINDING CHARACTERISTICS OF SNAKE AND CONE SNAIL VENOM POSTSYNAPTIC NEUROTOXINS: FURTHER STUDIES WITH A NON-RADIOACTIVE ASSAY

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B. G. STILES. Acetylcholine receptor binding characteristics of snake and cone snail venom postsynaptic neurotoxins: further studies with a non-radioactive assay. *Toxicon* **31**, 825–834, 1993.—The binding of postsynaptic neurotoxins from snake and marine cone snail (*Conus* sp.) venoms to nicotinic acetylcholine receptor (AchR) was investigated with an ELISA-based, non-radioactive assay. Three snake postsynaptic toxins from the long-chain group (*Naja naja kaouthia* cobratoxin, *Naja oxiana* neurotoxin I, *Bungarus multicinctus* α-bungarotoxin) and short-chain group (*Naja naja atra* cobrotoxin, *Naja oxiana* neurotoxin II, and *Laticauda semifasciata* erabutoxin b) were studied. Both types of snake postsynaptic toxins showed a dose–response with constant AchR (50 μg/ml) and varying toxin concentrations (50–0.035 μg/ml). The minimum detection limits of the assay for snake toxins ranged from 310 to 1240 ng/ml (40–160 pmole/ml), depending on the toxin. Unlike any of the short-chain toxins, long-chain toxins consistently bound less receptor and reached maximum absorbance levels with toxin concentrations of 10–50 μg/ml. Competition for AchR binding between cone snail postsynaptic neurotoxins (conotoxins GI, MI, SI) and α-bungarotoxin or cobrotoxin resulted in a dose–response. The postsynaptic conotoxins were uniformly better competitors for AchR binding with α-bungarotoxin than with cobrotoxin. Heat stability studies with neurotoxin I, erabutoxin b, or cobrotoxin revealed a loss in AchR binding activity with increasing temperature. α-Bungarotoxin heated at 90°C had increased AchR binding activity by 105%, relative to 25°C samples, but lost the majority of its binding activity after 100°C. The enhanced binding of heated α-bungarotoxin to AchR was specific, as evidenced by a competitive dose–response with unheated α-bungarotoxin, but heated toxin lacked any biological activity in the mouse lethal assay. When conotoxins GI or MI were heated at 100°C, there was no detectable loss in AchR binding activity, and only a slight decrease in mouse lethality.

INTRODUCTION

NUMEROUS postsynaptic neurotoxins from snake and marine cone snail (*Conus* sp.) venoms bind specifically to similar sites on the nicotinic acetylcholine receptor (AchR)

(CHANGEUX *et al.*, 1970; ISHIKAWA *et al.*, 1977; MCMANUS *et al.*, 1981). Although snake and cone snail postsynaptic neurotoxins share little sequence homology, there are three-dimensional similarities (HIDER, 1985; DUFTON *et al.*, 1989). Snake venom postsynaptic neurotoxins are classified as long-chain or short-chain toxins, based on mol. wt and number of disulfide bridges (LEE, 1979). A high degree of amino acid homology exists within, but not between, the long- and short-chain toxin groups (LEE, 1979). Snake postsynaptic neurotoxins have mouse LD_{50} values generally ranging from 50 to 150 μg toxin/kg (DUFTON and HARVEY, 1989).

Long-chain and short-chain, postsynaptic neurotoxins from snake venom have similar AchR binding K_ds of 10^{-10}–10^{-11} M, but noticeable differences exist (LEE *et al.*, 1972; CHICHEPORTICHE *et al.*, 1975). The short-chain toxins have higher on off binding rates for receptor and also fail to recognize peptide region 182-198 from the alpha subunit of *Torpedo californica* AchR, which is important for long-chain toxin binding (WEBER and CHANGEUX, 1974; CHICHEPORTICHE *et al.*, 1975; RUAN *et al.*, 1991). A previous study using the non-radioactive assay with three snake short-chain neurotoxins and one long-chain neurotoxin repeatedly showed less AchR binding by the latter (STILES, 1991).

The conotoxins isolated from cone snail venom have various pharmacological effects, but those that bind postsynaptically to AchR contain 13–15 amino acids and two disulfide bridges (MCMANUS *et al.*, 1981; CRUZ *et al.*, 1985; OLIVERA *et al.*, 1985; ZAFARALLA *et al.*, 1988). Postsynaptic conotoxins MI or GI are more lethal on a molar basis (mouse LD_{50}s of 5 and 12 μg/kg, respectively) than snake postsynaptic neurotoxins, even though the conotoxins have a lower, more reversible affinity for AchR (GRAY *et al.*, 1983; MCMANUS and MUSICK, 1985). Conotoxin SI also binds postsynaptically and is particulary potent in fish, but has little effect in mice (GRAY *et al.*, 1983; ZAFARALLA *et al.*, 1988). When conotoxin GI is adsorbed onto a microtiter plate in the non-radioactive receptor assay, there is little detectable binding to receptor, which is unlike any of the tested snake postsynaptic neurotoxins (STILES, 1991). However, conotoxin GI did show a good competitive binding effect in solution with AchR when cobrotoxin was adsorbed onto the plate wells. Lack of AchR binding by conotoxin GI adsorbed onto microtiter plates was probably due to the smaller size ($M_r \sim 1500$), relative to snake toxins ($M_r \sim 7000$–8000), and subsequent steric problems associated with receptor interaction.

This study further explores the AchR binding characteristics of postsynaptic neurotoxins from snake and cone snail venom in an ELISA-based, non-radioactive receptor assay. Comparisons are made between *in vitro* and *in vivo* assay results with different toxins.

MATERIALS AND METHODS

Preparation of nicotinic acetylcholine receptor (AchR)

AchR from the electric organ of *Torpedo californica* was purified as previously described (FROEHNER and RAFTO, 1979; STILES, 1991). All materials used to purify AchR were autoclaved or rinsed in 70% ethanol. Buffers contained 1 mM EDTA, 1 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride to inhibit protease activity. Purified AchR was stored in sterile glycerol (33% final concentration) at −70°C.

Toxins and protein determinations

Purified snake venom toxins (*Laticauda semifasciata* erabutoxin-b, *Naja naja atra* cobrotoxin, *Naja oxiana* neurotoxins I and II, *Naja naja kaouthia* cobratoxin, and *Bungarus multicinctus* α-bungarotoxin) were obtained from Natural Product Sciences (Salt Lake City, UT, U.S.A.) and the identity confirmed using amino-terminus sequencing by Dr Jim Schmidt in our Institute. Synthesized and purified conotoxins GI, MI, SI, and GIIIA were purchased from Sigma (St. Louis, MO, U.S.A.) or Bachem Bioscience (Philadelphia, PA, U.S.A.). All toxins were diluted in phosphate-buffered saline, pH 7.4 (PBS) and protein concentrations were determined with the Bradford dye binding assay (BRADFORD, 1976) from Bio-Rad (Richmond, CA, U.S.A.) using bovine gamma globulin as a standard.

Non-radioactive receptor assay

The assay was essentially done as previously described (STILES, 1991) except that guinea-pig anti-AchR sera was diluted 1 : 200 in PBS containing 0.1% Tween 20 and 0.1% gelatin (PBSTG). Goat anti-guinea-pig IgG conjugated to alkaline phosphatase (Sigma) was diluted 1 : 1000 with PBSTG. All buffers were freshly prepared before each experiment. Dose–response studies were done with varying amounts of toxin (100 μl of a 50–0.035 μg toxin/ml carbonate buffer, pH 9.6 solution) adsorbed overnight onto Dynatech Immulon II ELISA plates (McClean, VA, U.S.A.) at 4°C. Purified receptor was used at a 50 μg/ml PBSTG concentration (100 μl/well). Minimal detection limits of the assay for each toxin were determined by doubling the mean reading of the low toxin concentration equivalent to background. Each dilution of every toxin was done in quadruplicate and the mean of OD_{405} readings ± standard deviation (S.D.) calculated.

Competitive binding assays were accomplished with varying amounts of conotoxins (3–50 μg/ml PBSTG) in solution with AchR (50 μg/ml PBSTG) and plate-adsorbed cobrotoxin or α-bungarotoxin (10 μg/ml carbonate buffer).

The heat stability of snake toxins was assessed by ELISA with 50 μg toxin/ml PBS solutions heated in microfuge tubes for 30 min in a constant-temperature hot water bath. After cooling to room temperature, heated toxin samples were diluted with carbonate buffer to a 10 μg/ml concentration and 100 μl added to each ELISA well for overnight incubation at 4°C. The binding assay was then completed as previously described.

Aliquots (100 μl) of conotoxins GI or MI from 500 μg/ml PBS solutions were heated in a hot water bath for 30 min at a constant temperature. After cooling to room temperature, each conotoxin sample was diluted with 150 μl of PBSTG and mixed with 250 μl of a 100 μg AchR/ml PBSTG solution. The conotoxin–AchR mixture (100 μl) was added to ELISA wells previously adsorbed with 10 μg/ml solutions of cobrotoxin or α-bungarotoxin and the binding assay completed as previously described.

Mouse lethal assays

Male Swiss–Webster mice (24 ± 1.6 g) were each injected i.p. with 200 μl of an α-bungarotoxin (10 μg equaling ~ $3LD_{50}$)-PBS solution. A 1 ml aliquot of a 50 μg toxin/ml solution was heated in a 90°C water bath for 30 min and then cooled to room temperature before injection. Four mice were used for each heated and unheated toxin sample.

Heated samples (100°C) of conotoxins GI and MI were also tested using ~ $3LD_{50}$ (1.6 or 0.7 μg of conotoxin GI or MI, respectively) per mouse. Groups of five mice per conotoxin sample were injected i.p. with 200 μl of toxin–PBS solution per mouse.

RESULTS

Figures 1 and 2 show the AchR binding curves for snake postsynaptic neurotoxins in a non-radioactive receptor assay. Equivalent concentrations of the short-chain toxins (cobrotoxin, neurotoxin II, erabutoxin b) apparently bound more AchR than long-chain toxins (α-bungarotoxin, cobratoxin, neurotoxin I) as evidenced by the higher absorbance readings. The minimal detection limits of the assay were not uniform within a toxin group since cobrotoxin, neurotoxin II, and α-bungarotoxin were detected at the lowest concentration of 310 ng/ml, erabutoxin b was detectable at 620 ng/ml, and neurotoxin I or cobratoxin were detected at 1240 ng/ml.

Receptor binding characteristics were similar within a toxin group but differed between the short- and long-chain toxins. At 10–50 μg/ml concentrations, all of the long-chain toxins yielded maximal OD_{405} readings, in contrast to short-chain toxin readings which were still within the upper, linear range of the dose–response curve.

Competitive AchR binding between varying concentrations of conotoxins GI, MI, or SI and constant amounts of plate adsorbed cobrotoxin or α-bungarotoxin yielded a dose–response (Tables 1 and 2). Postsynaptic conotoxins inhibited the AchR binding of α-bungarotoxin better than cobrotoxin, suggesting AchR binding differences between the

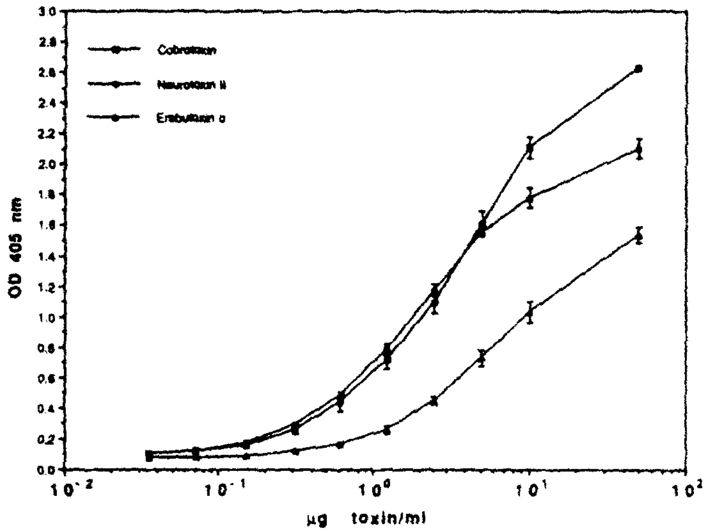

FIG. 1. RECEPTOR BINDING CURVES WITH SNAKE SHORT-CHAIN, POSTSYNAPTIC NEUROTOXINS (50–0.035 μg/ml) ADSORBED ONTO AN ELISA PLATE AND AchR (50 μg/ml PBSTG).
Bound receptor was detected with guinea-pig anti-AchR, anti-guinea-pig IgG conjugated to alkaline phosphatase, and finally substrate. All points represent the mean $OD_{405} \pm$ S.D. of four readings per sample.

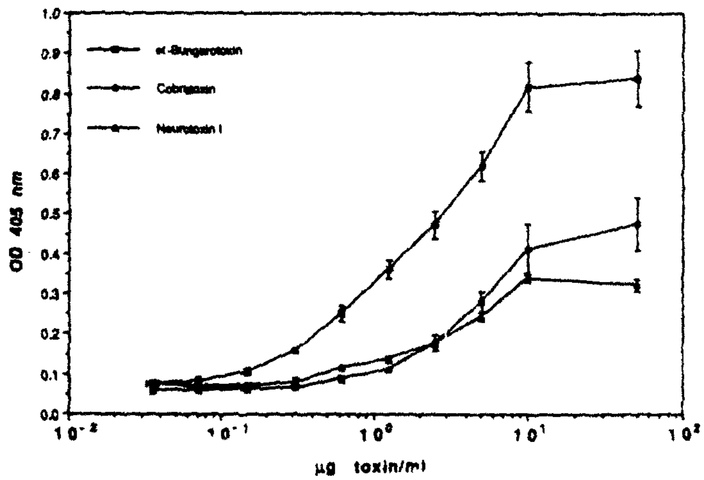

FIG. 2. RECEPTOR BINDING CURVES WITH SNAKE LONG-CHAIN, POSTSYNAPTIC NEUROTOXINS (50–0.035-μg/ml) ADSORBED ONTO AN ELISA PLATE AND AchR (50 μg/ml PBSTG).
Bound receptor was detected with guinea-pig anti-AchR, anti-guinea-pig IgG conjugated to alkaline phosphatase, and finally substrate. All points represent the mean $OD_{405} \pm$ S.D. of four readings per sample.

TABLE 1. COMPETITIVE RECEPTOR BINDING STUDIES WITH 10 μg COBROTOXIN/ml AND VARYING CONCENTRATIONS OF CONOTOXINS GI, MI, SI, OR GIIIA

Conotoxin competitor (μg/ml)	$OD_{405} \pm$ S.D. (% competition)* Conotoxin GI	Conotoxin MI	Conotoxin SI	Conotoxin GIIIA
50	0.800 ± 0.020 (50)	0.986 ± 0.056 (38)	1.067 ± 0.044 (33)	1.606 ± 0.055 (0)
25	0.885 ± 0.040 (44)	1.076 ± 0.031 (32)	1.120 ± 0.083 (29)	1.628 ± 0.046 (0)
12	1.088 ± 0.057 (31)	1.220 ± 0.042 (23)	1.230 ± 0.051 (23)	1.685 ± 0.055 (0)
6	1.218 ± 0.042 (23)	1.292 ± 0.035 (19)	1.371 ± 0.048 (14)	1.713 ± 0.133 (0)
3	1.298 ± 0.081 (19)	1.374 ± 0.066 (13)	1.459 ± 0.065 (8)	1.671 ± 0.048 (0)

*Control wells with cobrotoxin (10 μg/ml), AchR (50 μg/ml), guinea-pig anti-AchR serum (1 : 200), anti-guinea-pig conjugate (1 : 1000), and substrate with no conotoxin competitor had a mean OD_{405} reading ± S.D. of 1.588 ± 0.075. Negative control wells containing all of the above except AchR gave readings of 0.013 ± 0.009. The % competition was calculated by the equation: 1 − (mean of test readings/1.588).

TABLE 2. COMPETITIVE RECEPTOR BINDING STUDIES WITH 10 μg α-BUNGAROTOXIN/ml AND VARYING CONCENTRATIONS OF CONOTOXINS GI, MI, SI, OR GIIIA

Conotoxin competitor (μg/ml)	$OD_{405} \pm$ S.D. (% competition)* Conotoxin GI	Conotoxin MI	Conotoxin SI	Conotoxin GIIIA
50	0.192 ± 0.014 (68)	0.224 ± 0.002 (62)	0.212 ± 0.018 (64)	0.735 ± 0.004 (0)
25	0.156 ± 0.009 (74)	0.206 ± 0.016 (65)	0.223 ± 0.012 (62)	0.685 ± 0.010 (0)
12	0.202 ± 0.002 (66)	0.230 ± 0.010 (61)	0.258 ± 0.012 (56)	0.658 ± 0.014 (0)
6	0.226 ± 0.006 (62)	0.275 ± 0.022 (54)	0.331 ± 0.008 (44)	0.638 ± 0.015 (0)
3	0.261 ± 0.006 (56)	0.304 ± 0.013 (49)	0.417 ± 0.013 (30)	0.610 ± 0.026 (0)

*Control wells with α-bungarotoxin (10 μg/ml), AchR (50 μg/ml), guinea-pig anti-AchR serum (1 : 200), anti-guinea-pig conjugate (1 : 1000), and substrate with no conotoxin competitor gave a mean OD_{405} reading ± S.D. of 0.593 ± 0.044. Negative control wells containing all of the above except AchR gave readings of 0.013 ± 0.004. The % competition was calculated by the equation: 1 − (mean of test readings/0.593).

TABLE 3. AChR BINDING CHARACTERISTICS OF HEATED SNAKE POSTSYNAPTIC NEUROTOXINS

Temperature	$OD_{405} \pm$ S.D.* Cobrotoxin	Neurotoxin II	Erabutoxin b	Neurotoxin I	α-Bungarotoxin
25	1.976 ± 0.062	1.599 ± 0.035	1.045 ± 0.054	0.440 ± 0.047	0.759 ± 0.013
60	1.546 ± 0.049	1.558 ± 0.057	0.735 ± 0.042	0.308 ± 0.014	0.657 ± 0.026
80	1.432 ± 0.050	1.474 ± 0.069	0.681 ± 0.041	0.091 ± 0.009	0.669 ± 0.026
90	1.259 ± 0.074	2.608 ± 0.015	0.306 ± 0.029	0.095 ± 0.008	1.552 ± 0.059
100	0.235 ± 0.013	2.634 ± 0.144	0.084 ± 0.004	0.087 ± 0.004	0.171 ± 0.020

*All readings were in quadruplicate and the mean of OD_{405} readings ± S.D. calculated. Negative control readings for wells containing no toxin plus AchR and all other reagents were 0.091 ± 0.010.

conotoxins and snake short- or long-chain neurotoxins. Conotoxin GIIIA, which binds to voltage-sensitive sodium channels in muscle, was used as a negative control and did not block the binding of either snake toxin to AchR.

TABLE 4. EFFECTS OF HEATING CONOTOXINS GI OR MI (25 μg/ml) IN COMPETITIVE AchR BINDING ASSAY WITH COBROTOXIN OR α-BUNGAROTOXIN (10 μg/ml)

Temperature	Cobrotoxin (OD_{405} ± S.D.)* Conotoxin GI	Conotoxin MI	α-Bungarotoxin (OD_{405} ± S.D.)† Conotoxin GI	Conotoxin MI
25	1.118 ± 0.019	1.186 ± 0.064	0.326 ± 0.009	0.307 ± 0.015
60	1.088 ± 0.054	1.103 ± 0.088	0.338 ± 0.015	0.321 ± 0.023
80	1.050 ± 0.055	1.156 ± 0.023	0.324 ± 0.009	0.320 ± 0.024
90	1.085 ± 0.058	1.211 ± 0.069	0.299 ± 0.005	0.346 ± 0.023
100	1.158 ± 0.078	1.238 ± 0.037	0.365 ± 0.022	0.365 ± 0.037

*All readings were in quadruplicate and the mean of OD_{405} readings ± S.D. calculated. Control readings for cobrotoxin without any conotoxin inhibitor were 1.810 ± 0.077. Control well readings without any toxin plus AchR were 0.116 ± 0.012. The % competition of conotoxins GI and MI for AchR binding with cobrotoxin at 25°C was 38% and 34%, respectively.

†Control readings for α-bungarotoxin without any conotoxin inhibitor were 1.043 ± 0.038. The % competition of conotoxins GI or MI for AchR binding with α-bungarotoxin at 25°C was 69% and 71%, respectively.

The thermostability of snake and snail venom postsynaptic neurotoxins was investigated with the *in vitro* receptor binding assay (Tables 3 and 4). Cobrotoxin, erabutoxin b, and neurotoxin I had progessively less AchR binding activity after increasing heat treatment. Neurotoxin I was the most heat labile snake toxin with little receptor binding activity at 80°C or above. α-Bungarotoxin lost very little AchR binding activity when heated to 90°C and surprisingly had an increased AchR binding activity of 105% at 90°C, relative to 25°C samples of toxin. When α-bungarotoxin was heated at 100°C, most of the receptor binding activity *in vitro* was destroyed. The enhanced AchR binding of heated α-bungarotoxin was specific, as evidenced by competitive inhibition rates of 7%, 44%, and 61% with 100, 500, and 2500 ng/ml of unheated α-bungarotoxin competitor, respectively (data not shown). In contrast to the elevated AchR binding activity of 90°C-heated α-bungarotoxin, mouse lethal activity (~ 3 LD_{50}/mouse) was totally abolished (data not shown).

The increased AchR binding of neurotoxin II heated at 100°C was non-specific since unheated neurotoxin II (100–5000 ng/ml) did not compete for receptor binding (data not shown). Additionally, data from negative control wells containing heated neurotoxin II and all other reagents except AchR were unexpectedly similar to positive control wells.

The competitive AchR binding activity of 100°C-heated conotoxins GI or MI with unheated cobrotoxin or α-bungarotoxin adsorbed onto ELISA wells did not differ in AchR binding activity compared to 25°C samples (Table 4). The biological activity of either conotoxin GI or MI (~ 3 LD_{50}) heated at 100°C was slightly less (four of five mice died) compared to unheated toxin controls (five of five mice died).

DISCUSSION

Results from the non-radioactive AchR assay show a distinct difference between the binding curves of snake long- and short-chain postsynaptic neurotoxins. After a 1 hr incubation of plate adsorbed toxin with AchR (50 μg of each/ml) in the non-radioactive receptor assay, the three long-chain toxins had reached maximal absorbance levels, yet short-chain toxins, at the same toxin-AchR concentrations, had not. A previous study with snake postsynaptic neurotoxins showed that long-chain toxins are five to nine times less likely to dissociate from the receptor after binding compared to short-chain toxins (CHICHEPORTICHE *et al.*, 1975).

The apparent lower AchR binding activity of snake long-chain, postsynaptic neurotoxins in this assay, relative to short-chain toxins, might be increased with more lengthy incubation periods since long-chain toxins associate six to seven times more slowly with AchR than short-chain toxins (CHICHEPORTICHE *et al.*, 1975). Studies with *T. marmorata* electric organ homogenate suggest that the receptor binding equilibrium of snake short- or long-chain toxins is attained in ~16 hr (ISHIKAWA *et al.*, 1977). Molar concentrations of short- or long-chain toxins (~7000 and ~8000 M_r, respectively) used in the non-radioactive AchR assay differed by 13% but do not account for the dissimilarities in receptor binding activity.

One possibility for the different AchR binding profiles seen with short- and long-chain toxins is the amount of toxin actually adsorbed onto an ELISA well. Although equal concentrations of toxin were added per well, the number of molecules adsorbed onto an ELISA well in the proper binding orientation for receptor may differ. Toxin concentrations were determined with a Coomassie Brilliant Blue dye binding assay (BRADFORD, 1976) and variability between different toxins exists due to the number of reactive groups (primarily NH_3^+ found on lysine) per molecule of toxin (FAZEKAS *et al.*, 1963). The use of extinction coefficients and absorbance readings at 280 nm for each toxin would lead to a more accurate estimation of protein concentration. Unfortunately, published extinction coefficient values for all of the toxins studied are not available.

Previous AchR binding studies with cobrotoxin showed that only 30% of a 50 μg toxin/ml concentration had adsorbed onto the ELISA wells and only 1.8% of the toxin added per well was oriented properly for receptor binding (STILES, 1991). The structural differences which naturally exist between snake short- and long-chain neurotoxins (LOW and CORFIELD, 1986), and varying degrees of altered toxin conformations after adsorption onto an ELISA plate, likely play a large role in the types of AchR binding curves seen in the non-radioactive receptor assay with both classes of toxin.

The different receptor binding profiles of snake short- and long-chain postsynaptic neurotoxins may also be explained by recent AchR peptide studies (RUAN *et al.*, 1991). Snake toxins bind to AchR by contacting various peptide regions on the AchR α-subunit (RALSTON *et al.*, 1987; CONTI-TRONCONI *et al.*, 1990). The *Torpedo* α-subunit peptide 182-198 is an important contact region for long-chain toxins, like α-bungarotoxin and cobratoxin, but is not recognized by the short-chain toxins, erabutoxin b and cobrotoxin (NEUMANN *et al.*, 1986; RUAN *et al.*, 1991). Long-chain toxins may not readily associate with receptor, like the short-chain toxins, because more contact sites are needed for optimal binding and might be shown in this study with the uniformly lower readings of long-chain toxins, relative to short-chain toxins. After a snake toxin has reached binding equilibrium with AchR, the extra contact sites between a long-chain toxin–receptor complex could then confer more stability than a short-chain toxin–receptor complex.

Previous competition studies between conotoxins MI, SI, or GI, and ^{125}I-labeled α-bungarotoxin for homogenized *Torpedo* sp. electric organ tissue have shown that the order of binding competitiveness for receptor was MI > SI > GI (ZAFARALLA *et al.*, 1988). These findings do not correspond with mouse LD_{50} findings since conotoxin MI is ~2.5 times more lethal than conotoxin GI, while conotoxin SI has very little effect in mice (ZAFARALLA *et al.*, 1988). Additionally, conotoxin GI is ~2.5-fold more potent in cats than conotoxin MI (MARSHALL and HARVEY, 1990). In the non-radioactive receptor assay, competitive binding between postsynaptic conotoxins and either cobrotoxin or α-bungarotoxin for purified *T. californica* AchR resulted in a competitive order of GI > MI > SI. The conotoxin competition results from this study and ZAFARALLA *et al.* (1988) may differ because of dissimilar methods of protein estimation and/or source of AchR. It is known that the binding of α-bungarotoxin to AchR isolated from various sources may differ by 100-fold, presumably because of structural variation among AchR α-subunits caused by dissimilar amino acid sequences (OHANA and GERSHONI, 1990).

All postsynaptic conotoxins inhibited the AchR binding of α-bungarotoxin better than cobrotoxin, suggesting that receptor contact sites for conotoxins GI, MI, or SI are more similar with α-bungarotoxin than cobrotoxin. Additionally, snake long-chain toxins may be more conformationally altered than short-chain toxins when adsorbed onto an ELISA well, thus resulting in decreased binding avidity of the long-chain toxins for AchR. A loss in binding avidity for AchR by long-chain toxins may be interpreted by consistently lower receptor binding readings and a greater degree of competitiveness by the conotoxins, relative to results for the short-chain toxins.

There were no uniform receptor binding characteristics among heated short-or long-chain snake toxins, but conotoxins GI and MI were both very stable, with no detectable decrease in AchR binding activity and only a slight loss in mouse lethal potency even after heating at 100°C. The smaller size of the conotoxins with limited tertiary structure and disulfide bonds, relative to the snake toxins, probably accounts for the extraordinary heat stability. Of notable interest among the snake toxins was the increased, specific binding of 90°C-heated α-bungarotoxin to AchR, which suggests a conformational change in the toxin allowing for better interaction with the *Torpedo* receptor. The mouse lethal effects of 90°C-heated α-bungarotoxin were not concomitantly enhanced, and actually abolished, relative to unheated toxin. Discrepancies between *in vitro* binding of snake postsynaptic toxins to *Torpedo* AchR and mouse lethality have been seen before (ISHIKAWA *et al.*, 1977).

In contrast to α-bungarotoxin, the increased AchR binding activity of 100°C-heated neurotoxin II was not specific as there was no competition with unheated toxin. Control wells containing all reagents used in the assay including heated neurotoxin II, but no AchR, inexplicably gave false positive results.

Upon heating the snake toxins, conformational changes within the molecule could impact upon the adsorption of toxin onto ELISA wells and ultimately the amount of bound receptor. Heat stability studies with the snake toxins were done by adsorbing heated toxin onto the wells. Another experimental approach would involve heated snake toxin as an AchR binding competitor in solution, like the conotoxin studies, with unheated toxin adsorbed onto the wells. This would address the question of whether decreased receptor binding by heated snake toxins might be due to less adsorption of properly oriented toxin onto the ELISA wells.

Besides information found in this communication, the non-radioactive receptor assay has been useful in this laboratory for determining the ability of monoclonal antibodies to block cobrotoxin binding to AchR and correlating these results with protection in mice (STILES *et al.*, 1991). Presumably any proteinaceous, or non-proteinaceous, molecule which binds to the nicotinic acetylcholine receptor could be used in this assay. This could be done by either adsorbing the suspect compound onto ELISA wells, like the snake toxins, or using the molecule in solution as a competitor for AchR, like the conotoxins.

The results from an *in vitro* AchR binding assay must be interpreted carefully, especially when trying to correlate toxin binding to *Torpedo* AchR with lethal effects in mammals. Although AchR is a relatively conserved protein among various animal species, amino acid sequences differ sufficiently to account for varying *in vivo* (mouse lethality) and *in vitro* (AchR binding) results (BURDEN *et al.*, 1975; PETERSON, 1989; BARCHAN *et al.*, 1992). Obviously, the most reliable correlation between an *in vivo* and *in vitro* assay would occur when AchR binding assays are done with receptor isolated from the same animal species used for lethal studies.

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